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13. ABSTRACT *(Maximum 200 Words)*

The purpose of this proposal is to isolate estrogen-responsive genes in human breast cancer cells. Cisplatin and formaldehyde cross-link protein to DNA, formaldehyde also cross-links protein to protein. Chromatin immunoprecipitation (ChIP) using anti-estrogen receptor (ER) antibodies was applied to isolate ER-bound DNA in situ. This protocol should isolate all ER-bound DNA fragments. Our first specific aim was to establish a protocol to isolate DNA bound in situ to ER, and characterize this ER-bound DNA. We have carried out chromatin immunoprecipitation procedure using an anti-ER monoclonal antibody and an ER+ human breast cancer T5 cell to isolate ER-bound DNA. Southern blot analysis shows that ER-bound DNA contains ER-responsive genes (such as PR, ER, pS2 and c-myc), but not control λ-DNA. The pS2 gene promoter was analyzed using electrophoretic mobility shift assay, and three Sp1/Sp3 binding sequences were identified. A high resolution mapping protocol to find location of ER along the pS2 gene promoter was developed. PCR results indicated that estradiol increases ER binding to pS2 promoter. We are testing the optimum conditions to construct an ER-bound genomic DNA library.

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5. INTRODUCTION

A. ER and Breast Cancer

The proliferation of cells in hormonally dependent tumors is regulated by the expression of ER responsive genes. The first step in the mechanism by which estrogens stimulate growth and antiestrogens inhibit growth of many ER+ human breast cancer cells is the direct binding of ligand to ER. ER is a DNA-binding protein that can transcriptionally regulate target genes by directly interacting with the gene's promoter elements (Davie et al., 1999; Murphy, 1998). *In vitro* assays have shown that ER binds to an estrogen response element (ERE) consisting of a palindromic DNA sequence. ER interaction with the ERE results in structural changes in both the ER protein and ERE DNA (Wood et al., 1998). *In situ*, the ERE may be associated with a nucleosome, as is the case for the ERE of the pS2 gene (Sewack and Hansen, 1997). Thus, ER-induced alterations in ERE DNA conformation may result in alterations in nucleosome structure (Bishop et al., 1997). ER can interact synergistically with several transcription factors and bind to a half palindromic ERE (Beato, 1989; Gorski et al., 1993; Schule et al., 1988; Meyer et al., 1989; Kato et al., 1992; Beato et al., 1996; Krishnan et al., 1994; Porter et al., 1996). Most of the characterized EREs of breast cancer estrogen responsive genes have ER and Sp1 binding sites (Xie et al., 1999; Qin et al., 1999). The regulatory regions of these genes (e.g. retinoic acid receptor α1, cathepsin D, *c-fos*, adenine deaminase, hsp27, and insulin-like growth factor-binding ptorein-4 genes) have an ERE (1/2) $(N)_x$ Sp1 (a half-site ERE positioned next to a Sp1 site) (Duan et al., 1998; Krishnan et al., 1994; Porter et al., 1996; Qin et al., 1999; Samudio et al., 2001; Sun et al., 1998; Vyhlidal et al., 2000; Wang et al., 1999; Xie et al., 1999; Xie et al., 2000). The *c-myc* gene lacks a consensus ERE, however it is possible that this estrogen-induced gene is regulated by an ER/Sp1 interaction (Dubik and Shiu, 1992; Miller et al., 1996). In contrast to classical ER-ERE interactions several of the genes with the ERE (1/2) $(N)_x$, Sp1 do not require ER to bind to DNA (Xie et al., 1999; Miller et al., 1996). In this context, ER acts as a coactivator, which binds to Sp1 bound to a GC-rich motif. The pS2 gene, a human breast cancer prognostic marker, has a

consensus ERE in the 5'-flanking promoter (Jeltsch et al., 1987; Lu et al., 2001). Here we report that few Sp1 binding sites close to ERE in this region have identified.

A variety of approaches currently used by molecular biologists will be applied to identify the genes that are regulated by estrogens. Identifying these genes increases our understanding of how estrogens work in promoting the growth of breast cancer cells.

B. RESEARCH OBJECTIVES

The goals of this research are to isolate, characterize and identify the ER-bound DNA fragments *in situ*. After ER-bound genomic DNA is isolated, an ER-bound genomic DNA library will be constructed. This protocol should isolate ER responsive promoters and also ER associated genomic DNA that may have a structural function in chromatin. In our statement of work in the first year we had set out three tasks as follows:

Task 1	Months 1-15	Large batch tissue culture of MCF-7 cells and MCF-7 cells that stably express epitope-tagged ER under the control of the tetracycline-on system.
Task 2	Months 2-15	Establish a protocol to isolate DNA bound in situ to ER and epitope-tagged ER in above mentioned cell lines. Cells will be incubated with cisplatin or formaldehyde, DNA fragmented and ER-DNA isolated by immunoprecipitation. Determine efficiency of ER cross-linking to nuclear DNA in immunoblotting experiments.
Task 3	Months 9-18	Characterization of ER-DNA by Southern and

Northern blot analysis. Develop high resolution mapping protocol to find location of ER along the c-myc promoter.

In this report we show the progress that we have made in achieving these tasks. We have tested a commercially available anti-ERα monoclonal antibody to immunochemically stain and immunoprecipitate wild-type ER, and we found that this antibody immunochemically stained wild-type ER with very low background and is very good for immunoprecipitation of wild-type ER in cells. When we proposed this project, we planed to use anti-HA, anti-Histidine or anti-GFP antibodies to immunoprecipitate an epitope-tagged-GFP-ER that is expressed in a MCF-7 cell line. Since we were able to quantitatively immunoprecipitate wild-type ER, we decided it was not necessary to use the tagged ER construct.

The pS2 promoter contains a consensus ERE sequence. ER could directly bind or indirectly bind DNA in pS2 promoter. As many ER-responsive genes have Sp1 sites to recruit ER bound in ERE, we examined the pS2 promoter for Sp1 sites. We have analyzed the Sp1 motifs in this region using electrophoretic mobility shift analysis, and identified three Sp1/Sp3 binding sequences close to ERE. We developed a high resolution mapping protocol to find location of ER along the pS2 promoter.

The chromatin immunoprecipitation (ChIP) protocol was used to successfully isolate ER-bound DNA fragments. Our PCR results show that ER differentially binds to the pS2 promoter of T5 cells treated with or without estradiol. This result is identical with Evans' report (Chen et al., 1999).

We have two different ways to identify ER-bound DNA sequences. We will first clone the ChIP isolated ER-bound genomic DNA fragments into a vector to construct a genomic library. Then, the clones will be sequenced, DNA sequences will be identified by the Blast search program. Alternatively, a rapid method to isolate and identify ER response genes could be designed. The ER-bound DNA fragments should include EREs of the promoters and/or enhancers of estrogen responsive genes. These sequences should be expressed in ER+ breast cancer

cells. We could use these ER-bound DNA to select by hybridization cDNA and to probe a human cDNA microarray to identify ER responsive genes. To test this idea, ChIP isolated ER-bound DNA was labeled and hybridized with known ER-responsive cDNA sequences: progesterone receptor (PR), estrogen receptor (ER), c-myc, pS2, d-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and glucose-6-phosphate dehydronase (G6PD) [Monet et al.,1987; Revillion et al., 2000; Zou et al.,1998]. Dot blot results showed that our ER-bound DNA could hybridize with these ER responsive cDNA, but did not hybridize with control λ-DNA. Human cDNA microarray analysis will soon be performed to identify ER-associated cDNA. Recently, the optimum conditions of digestion, ligation and cloning of ER-bound DNA fragments will be determined. After finishing this procedure, we will obtain an ER-associated genomic DNA library.

6. BODY REPORT

A. EXPERIMENTAL METHODS

i. Cell Culture

Human breast cancer T5 cells (ER positive and hormone dependent), MDA MB 231 cells (ER negative and hormone independent) were grown as described previously (Miller et al., 1996; Samuel et al., 1998). MCF-7 clone 11 cells that stably express epitope-tagged ER under the control of the tetracycline-on system were grown and doxycycline induced as described previously (Htun et al., 1999).

ii. Cisplatin and formaldehyde cross-linking cells

Cisplatin cross-linking condition was described before (Samuel et al., 1998). T5 cells were incubated with 10 mM cisplatin in Hanks buffer containing sodium acetate instead of sodium chloride (5.4 mM KCl, 0.3 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 4.2 mM $NaHCO_3$, 1.3 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.6 mM $MgSO_4$, 5.6 mM D-glucose, 137 mM Na.acetate) at 37 °C for 2 hours. After removing cisplatin, the cells were washed twice with TNM buffer (100 mM NaCl, 300 mM sucrose, 10 mM Tris-HCl, pH8.0, 2 mM $MgCl_2$, 1 % thiodiglycol), and harvested.

Formaldehyde cross-linking of cells was performed as previously described (Spencer et al., 2001). Formaldehyde was directly added to the medium to a final concentration of 1%. The cells were Incubated at 25 °C for 8 minutes. After cross-linking, the cells were washed with PBS twice and harvested.

Hydroxylapatite (HTP) was used to isolate DNA. Only free DNA and DNA with cross-linked protein can bind to HTP. The HTP resin was washed with lysis buffer to remove RNA and proteins not cross-linked to DNA. To reverse the cross-linking, HTP was incubated in thiourea for cisplatin cross-linked cells, or incubated at 65 °C for 4 hours for formaldehyde cross-linked cells.

iii. Chromatin Immunoprecipitation (ChIP)

Immunoprecipitation of chromatin fragments with anti-ER mouse monoclonal antibody (Novocastra Laboratories LTD, UK) was performed as described previously (Sun et al. 2001) with modification. Briefly, the cross-linked cells were

washed twice with PBS containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and proteinase inhibitor cocktails (Roche). The cells were resuspended in Lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1) and incubated on ice for 10 min. The cells were sonicated with 4 sets of 10 second pulses at 30% output. Under this condition, the DNA length ranges from 200 to 1000 base pairs. After a brief centrifugation, the supernatant was diluted to 2 A_{260}/ml with Dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.1, 167 mM NaCl). Five µl of anti-ERα mouse monoclonal antibodies were added in 2 A_{260}/ml of chromatin fraction, and incubated overnight at 4 °C. Approximately 50 µl of protein A agarose was added, and incubated at 4 °C for 2 hours. The beads were washed once with Low Salt Wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 150 mM NaCl), High Salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 500 mM NaCl), LiCl buffer (0.25M LiCl, 1% NP-40, 1% deoxycholate, 1mM EDTA, 10 mM Tris-HCl, pH 8.1) and twice with TE buffer, pH 8.0. After elution with Elution buffer (1% SDS and 0.1 M $NaHCO_3$), DNA was reversed in 0.2 M NaCl at 65 °C for 2 hours in formaldehyde cross-linked cells. DNA fragments were extracted with phenol / chloroform and precipitated with ethanol.

iv. PCR

DNA fragments isolated by ChIP or from the input were used as a template in PCR reactions. Primers were designed covering the human pS2 promoter region: pS2-U (GACGGAATGGGCTTCATGAGC) and pS2-L (GATAACATTTGCCTAAGGAGG). PCR was performed as described previously (Sun et al., 1999).

v. Western Blot Analysis

Western blot analysis was carried out as described previously (Sun et al., 1996b). Mouse monoclonal antibodies against human ERα (Novocastra Laboratories Ltd) were used.

vi. Dot blotting

Two μg of cDNA fragments from human PR, ER, pS2, c-myc, GAPDH, G6PD and λ-DNA were denatured and dotted on the Hybond membrane (Amersham). DNA isolated by ChIP was labelled with α-^{32}P dCTP (NCI) using Random Prime Kit (GIBCO). Dot blotting was performed as described previously (Delcuve and Davie, 1989). The radioactivity of the dots was detected and analysed by PhosphoImager Station (BioRad).

vii. Electrophoretic Mobility Shift Analysis (EMSA)

The electrophoretic mobility shift analysis was performed as previously described [Sun et al, 1996]. Oligonucleotides that contained Sp1 motif were synthesized,

5'-GAGCTCCTTCCCTTCCCCCTGCA-3' (P1-U),
5'-TTGCAGGGGGAAGGGAAGGAGCT-3' (P1-L),
5'-GTCAGGCCAAGCCTTTTTCCGGCC-3' (P2-U),
5'-TGGCCGGAAAAAGGCTTGGCCTGA-3' (P2-L),
5'-GTTACCCTGGCGGGAGGGCCTCTC-3' (P3-U),
5'-TGAGAGGCCCTCCCGCCAGGGTAA-3' (P3-L),
5'-GATGACCTCACCACATGTCGTCTC-3' (P4-U),
5'-TGAGACGACATGTGGTGAGGTCAT-3' (P4-L),
5'-GGGATCCCCGGGCCTCCTTAGGCA-3' (P5-U),
5'-TTGCCTAAGGAGGCCCGGGGATCC-3' (P5-L).

In brief, 10 μg of nuclear extract was incubated with or without 1 μg of anti-Sp1 or anti-Sp3 antibodies (Santa Cruiz) for 10 min, then ^{32}P-dCTP labelled oligonucleotides were added in the reaction and incubated for 30 min. The radio-labelled DNA-protein complexes were resolved on polyacrylamide gel and exposed to X-ray films.

B. ASSUMPTIONS

The strategy of these studies is to cross-link ER to DNA *in situ* in human breast cancer cells with cisplatin or formaldehyde and then immunoprecipitate the ER cross-linked DNA by anti-ER antibodies. Our strategies will lead to the

construction of libraries of DNA sequences associated with ER *in situ*, and identify all ER target DNA in human breast cancer cells.

We hypothesise that most estrogen-induced genes will have consensus EREs that bind directly to ER; on the other hand, ER might indirectly bind to DNA fragments through other factors. After cross-linking, chromatin immunoprecipitation could bring down all ER associated DNA fragments. We will construct the library containing all DNA sequences that were associated with ER.

C. RESULTS AND DISCUSSION

i. Selection of Cell Lines and Antibodies

The critical factor for a successful ChIP protocol for the isolation of ER-bound DNA is an antibody that efficiently and specifically immunoprecipitates ER. Before writing this proposal, an anti-ER antibody with the specificity was not available. In our lab, we isolated a MCF-7 clone 11 cell line that expresses an inducible epitope-tagged-GFP-ER. After doxycycline induction, this cell expresses $(His)_6$-(HA)-GFP-ER (Htun et al., 1999). In the proposal, we designed a protocol to immunoprecipitate this epitope-tagged-GFP-ER by anti-histidine, anti-HA, or anti-GFP antibodies.

Since this project was started, a commercially available human ERα monoclonal antibody (NovoCastra Laboratories Ltd, UK) was tested. The total cell lysate from T5 cells treated with or without estradiol were immunochemically stained using this antibody. Figure 1 A shows that this antibody is good for Western blotting analysis with a low background. The efficiency of immunoprecipitation of this antibody was tested too. Total cell lysate from T5 cells was incubated with anti-ER antibody. Both the bound and unbound fractions were immunochemically stained with anti-ER antibody. Figure 1 B shows that the anti-ERα antibody could immunoprecipitate wild-type ER with a high efficiency.

Since the commercially available antibody is specific and efficient to immunoprecipitate wild-type ER, we decided not to apply the epitope tagged strategy. In this study, a breast cancer T5 cell (ER positive and hormone dependent) and anti-ERα monoclonal antibody was used.

ii. Analysis of cross-linking

T5 cells cross-linked with cisplatin or formaldehyde were lysed by sonication. The cell lysate was added to a hydroxylapatite (HTP) column to isolate DNA. Only free DNA and DNA with cross-linked protein can bind to HTP. The HTP resin was washed with lysis buffer to remove RNA and proteins not cross-linked to DNA. To reverse the cross-linking, HTP was incubated in thiourea for cisplatin cross-linked cells, or incubated in the presence of 0.2 M NaCl at 65 °C for 4 hours for formaldehyde cross-linked cells. Proteins released from HTP were immunochemically stained with anti-ER antibodies. Fig.2 shows that ER was cross-linked to DNA by cisplatin and formaldehyde. This result is Identical to our previous experiments (Samuel et al., 1998). We have shown that the different concentrations (from 35 μM to 1 mM) of cisplatin cross-linked protein to DNA *in situ* to different extents (Samuel et al., 1998). We do not want to cross-link for long time, as this will be lower our yield of DNA from the lysed and sonicated cells.

Cisplatin cross-links protein to DNA at a distance of 4 Å [Lippard et al, 1982], indicating that DNA cross-linked by cisplatin has ER directly bound to DNA sequences. Formaldehyde cross-links protein to DNA and proteins to proteins (Orlando et al., 1997; Dedon et al., 1991), indicating that DNA cross-linked by formaldehyde is a result of ER directly and/or indirectly binding to DNA sequences. We cannot calculate the accurate efficiencies of cross-linking, since there are a number of ER molecules that do not bind to DNA. The data show that there are 1.86×10^5 ER molecules in breast cancer cell far in excess of the total number of genes (3×10^4) (Coutts et al., 1996). Most ER molecules may not be associated with DNA in the breast cancer cells. The data also indicated that in the presence of ligand, such as estradiol, the ER is tightly bound to nuclear matrices.

iii. Analysis of pS2 5'-flanking promoter

Human pS2 gene is an ER regulated gene in breast cancer MCF-7 cells (Berry et al., 1989; Kim et al., 2000). In the 5'-flanking region of pS2 gene, there is an ERE sequence (AGGTCACGGTGGCC). The recent data indicated that the interaction of ER with Sp1 regulates most ER responsive promoters in breast cancer cells. Does ER-Sp1 complex also occur in pS2 promoter? First, we searched for possible Sp1 binding sequences in 5'-flanking region of pS2 gene with TFSEARCH program, and five potential Sp1 binding sequences around ERE were found (Table 1). Second, the electrophoretic mobility shift analysis was used to determine if these potential Sp1 binding sites bound Sp1 and/or Sp3 in vitro. The oligonucleotides containing these potential Sp1 binding sites were synthesized, called P1 to P5, and labeled with P^{32}-dCTP. The nuclear extract from T5 cells was incubated with or without anti-Sp1 or anti-Sp3 antibodies and P^{32} labeled oliogonucleotides. The DNA-protein complexes were resolved on polyacrylamide gels. The antibody supershift assay was used to check specific protein-DNA complex. If the protein in the complex was recognized by the antibody, it could form a DNA-protein-antibody complex that will move slowly through the gel to form supershifted bands with larger mass. If the protein in the complex is not recognized by the antibody, the DNA-protein complex will not be affected and the shifted bands will not change. The EMSA result indicates that P1, P2 and P3 could bind Sp1 or Sp3 in vitro, however P4 and P5 did not (Fig. 3). In the promoter of pS2 gene, there is a consensus ERE sequence and three Sp1/Sp3 binding sequences (Fig.5 A). Based on this experiment and Dr. Safe's reports, we used the pS2 promoter to identify ER-bound DNA *in situ*.

iv. Isolation of the Nuclear DNA Bound to ER

We have applied the ChIP protocol to isolate ER-bound chromatin from human breast cancer T5 cells. The formaldehyde cross-linked cells were lysed and sonicated. DNA extracted from the cell lysate was analysed. Under the above mentioned conditions, DNA sizes were between 1kb to 200 bp (Fig. 4) which is good for immunoprecipitation and PCR analysis. ER-bound DNA fragments were

immunoprecipitated by anti-ER antibodies. After reversing and precipitating, the ER-bound DNA was used as a template in PCR to check for presence of ER responsive promoters.

Two primers covering the pS2 5'-flanking region were designed to test ChIP DNA. DNA isolated by ChIP or directly (as Input) from T5 cells treated with or without estradiol (10nM for 30 minutes) was used as a template. PCR results showed that estradiol significantly increases the association of ER with the pS2 promoter ERE in T5 cells and, as expected, no pS2 5'-flanking DNA was detected in ER negative MDA MB 231 cells (Figure 5 B). This result indicates that the ChIP procedure and anti-ER antibodies could be used to isolate ER-bound DNA *in situ*.

v. Analysis of ER-bound DNA

Our final goal is to isolate and identify the DNA sequences bound to ER in human breast cancer cells. ER-bound genomic DNA fragments were randomly cut by sonication and their sizes were between 1 kb to 200 bp. A rapid method to identify these genes is to couple the ChIP assay with cDNA microarrys. Some, if not all, ER will be bound to the EREs of hormone responsive genes which should be expressed in T5 cells.

To test the feasibility of this approach, we determined the ability of ER-bound genomic DNA to hybridize to cDNA of estrogen responsive genes. Dot blotting analysis was performed to test this idea. Two μg of full length cDNAs, PR, pS2, c-myc, ER, GAPDH and G6PD, expressed in human breast cancer cells, and λ-DNA, as negative control, were dotted into Hybond N+ membrane. The membrane was hybridized with ^{32}P-labeled ER-bound DNA fragments isolated by ChIP. Fig. 6 shows that ER-bound DNA could directly hybridize to those ER-responsive cDNA, but not with control λ-DNA. This result indicates that ER-bound DNA could be used to identify some of ER responsive genes *in situ*. This experiment will be repeated using another negative control cDNA, cyclophilin D that is not an ER responsive gene (private communication).

D. RECOMMENDATIONS

Progress in the past year is on target with our proposed Statement of Work. For this experiment, we have tested human breast cancer ER positive T5 cell line and anti-ERα monoclonal antibodies for ChIP protocol. The ChIP procedure was performed using an anti-ERα monoclonal antibody. ER-bound DNA fragments were tested by PCR. The result shows that the antibodies and ChIP protocol work well for this experiment. We have tested the hybridization of ChIP-DNA with cDNA of ER responsive genes, such as PR, ER, c-myc, pS2, GAPDH and G6PD. In the next procedure, we will identify ER responsive genes with a human cDNA microarray; and clone the ER-bound DNA to construct a genomic DNA library of ER-bound DNA fragments.

7. KEY RESEARCH ACCOMPLISHMENTS

Isolation and characterization of ER-bound DNA from ER positive and hormone dependent human breast cancer T5 cells.

8. REPORTABLE OUTCOMES:

A. Abstracts:

Differential distribution of Sp1 and Sp3 in human breast cancer cells, 2001 AACR-NCI-EORTC International Conference on Molecular Targets and Cancer Therapeutics.

B. Presentations:

2001 FASEB Summer Research Conference on Nuclear Structure and Cancer

C. Publications:

1. Spencer VA and Davie JR, Isolation of proteins cross-linked to DNA by cisplatin, The protein protocol handbook, in press.
2. Spencer VA and Davie JR, Isolation of proteins cross-linked to DNA by formaldehyde, The protein protocol handbook, in press.

9.CONCLUSIONS

In ER+ human breast cancer cell, ER directly or indirectly binds to DNA in ER-responsive genes. Cisplatin could cross-link protein to DNA, and formaldehyde could cross-link protein to protein and protein to DNA. DNA fragments isolated by ChIP from cisplatin cross-linked cells contain ER directly bound sequences, and DNA from formaldehyde cross-linked cells should contain both directly and indirectly ER bound sequences. We are applying a chromatin immunoprecipitation assay with anti-ER monoclonal antibodies to isolate ER-bound DNA fragments from formaldehyde or cisplatin cross-linked cells. PCR results show that ChIP protocol and anti-ER monoclonal antibodies successfully perform to isolate ER-bound DNA *in situ*.

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9.APPENDICES

Table 1 Oligonucleotides of Sp1 in pS2 promoter

Sp1 consensus sequence	G/T	G/A	G	G	T/A/C	G/T	G/A	G/A	G/T
P1	A	G	G	G	A	A	G	G	G
P2	C	G	G	T	T	C	G	G	A
P3	C	G	G	G	A	G	G	G	C
P4	G	G	T	G	T	G	G	T	G
P5	G	G	G	G	C	C	C	G	G

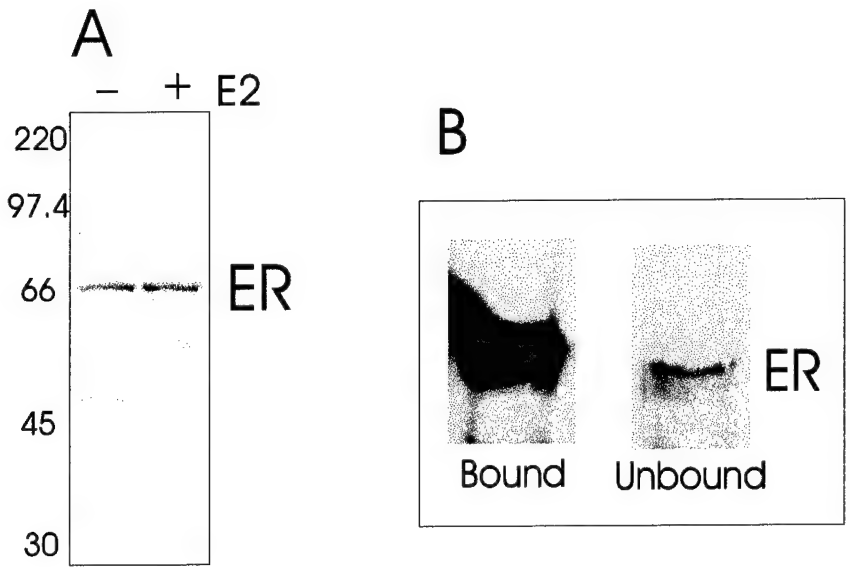

Fig.1 Western blotting analysis using anti-ERα monoclonal antibodies. Cell lysate from T5 cells with or without E2 treatment was loaded on 10% SDS polyacrylamide gel and immunochemically stained with anti-ERα monoclonal antibodies (A). T5 cell lysate was immunoprecipitated by anti-ERα monoclonal antibodies. Bound and unbound fractions were loaded on 10% SDS polyacrylamide gel and immunochemically stained by anti-ERα antibodies (B).

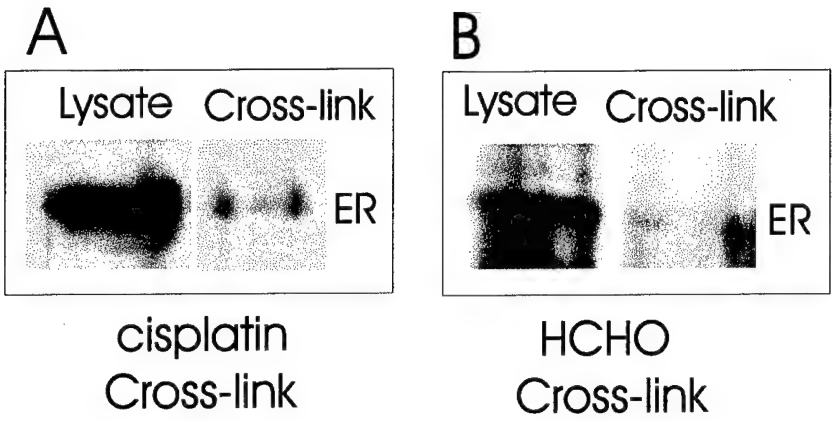

Fig. 2 Cross-linking analysis. T5 cells were incubated with 1mM cisplatin for 2 hours at 37°C or incubated with 1% formaldehyde for 8 min at 25 °C. The cells were lysed by sonication. The cell lysate was loaded on hydroxylapatite to isolate DNA and DNA with cross-linked proteins. The protein-DNA cross-links were reversed by incubation in thiourea for cisplatin cross-linking or by incubation at 65 °C for formaldehyde cross-linking. Cell lysate and cross-link fractions were loaded on 10% SDS polyacrylamide gel and immunochemically stained with anti-ERα antibodies.

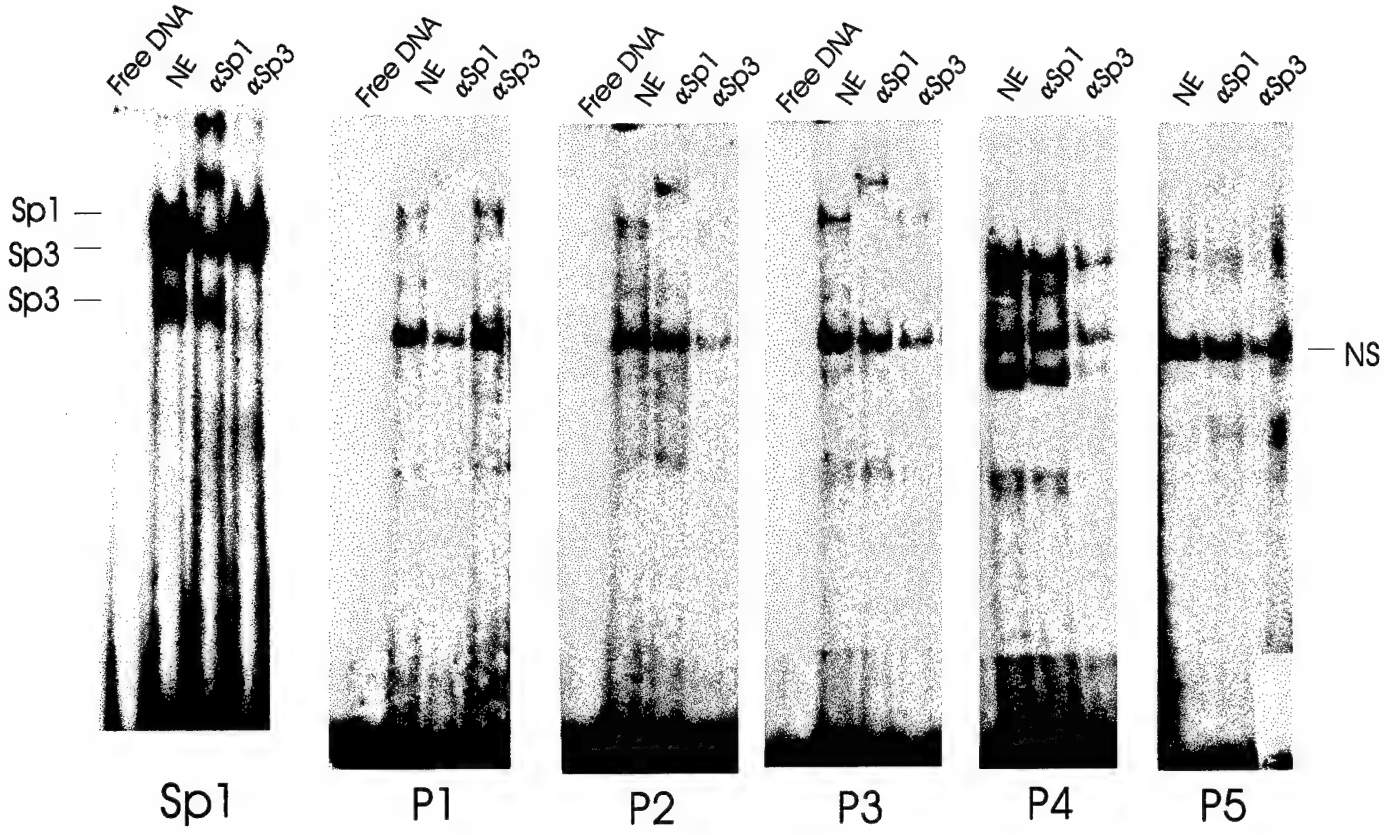

Fig.3 Electrophoretic mobility shift analysis of 5'-flanking region of pS2 gene. The oligonucleotides (see Table 1) were synthesized and end labeled with ^{32}P-dCTP. Ten μg of nuclear extract from T5 cells was incubated with or without 1 μg of anti-Sp1 or anti-Sp3 antibodies for 10 min, then with 10^4 dpm radiolabeled oligonucleotides. The DNA-protein complexes were resolved on the polyacrylamide gel and exposed to film. Sp1 and Sp3 are DNA-protein complexes, NS is non-specific binding.

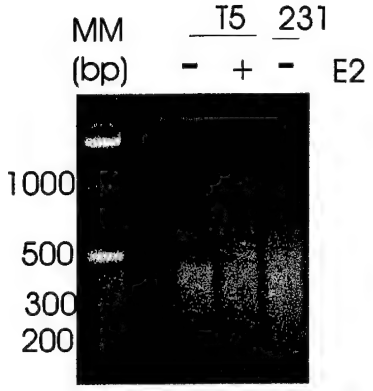

Fig. 4 T5 cells with or without E2 treatment and MDA MB 231 cells were lysed by sonication. DNA was extracted from chromatin by phenol / chloroform, loaded on 1% agarose gel, and stained with ethidium bromide.

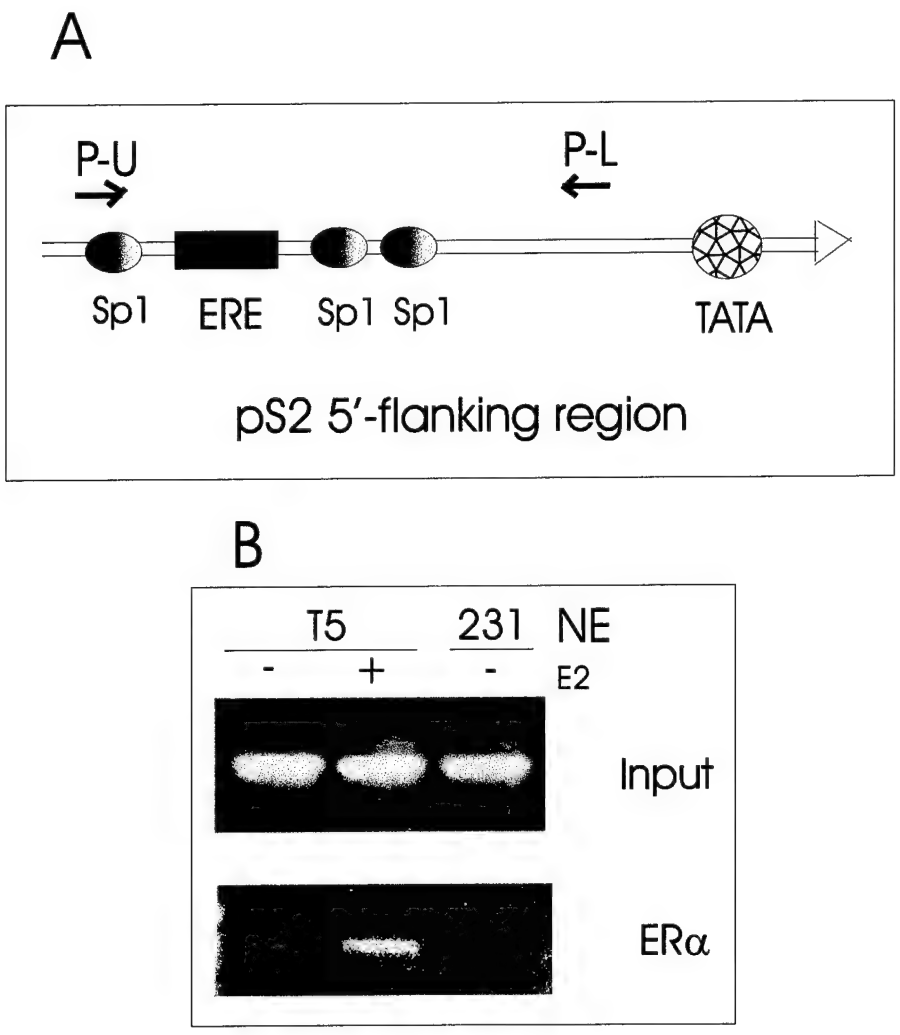

Fig. 5 PCR analysis of chromatin immunoprecipitated DNA by anti-ERα monoclonal antibodies. Chromatin from T5 (+/- E2) and MDA MB 231 cells were immunoprecipitated by anti-ERα monoclonal antibodies. Two primers (P-U and P-L), covered three Sp1 binding sites and ERE sequence in pS2 5'-flanking region, were designed (Panel A). Two hundred ng of ChIP DNA was used in each PCR reaction as template and 1 pM of pS2 primers were added in 50 μl of reaction. Twenty μl of reaction was loaded on 1% agarose gel and stained with ethidium bromide (Panel B).

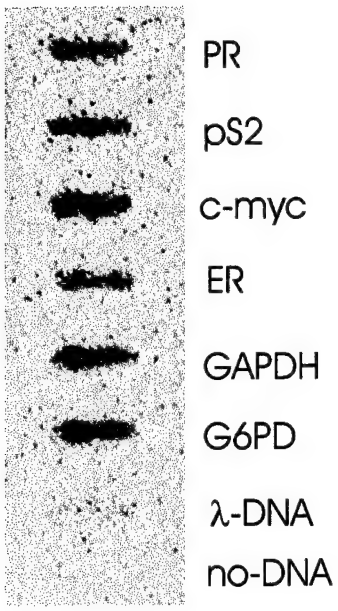

Fig. 6 Analysis of ER-bound DNA. DNA fragments from chromatin immunoprecipitation with anti-ERα monoclonal antibodies were random labeled with ^{32}P-dCTP as probe. Two μg of cDNA fragments from human genes (PR, pS2, c-myc, ER, GAPDH and G6PD) and -λDNA were dotted on nylon membrane and hybridized with the labeled probe.

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Differential distribution of Sp1 and Sp3 in human breast cancer cells

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Sp3 is a member of the Sp1 transcriptional factor family that plays an important role in gene transcription and regulation. The PS2 gene is actively expressed in human breast cancer cells. The differential distribution of Sp1 and Sp3 in T5 human breast cancer (estrogen receptor positive and hormone dependent) cells was investigated in this study. T5 cells were incubated with cisplatin for two hours to cross-link protein to DNA *in situ*, and the nuclear matrix proteins cross-linked to DNA were isolated. The DNA-binding nuclear matrix proteins were analysed by Western blotting with anti-Sp1 and anti-Sp3 antibodies. Both Sp1 and Sp3 are associated with DNA *in situ*. Triton X-100 extraction of nuclei was performed to analyze the subcellular distribution of Sp1 and Sp3. The majority of Sp3 was tightly bound to the nuclear matrix, while approximately 40% of Sp1 was released from the nuclei after Triton treatment. Furthermore, using chromatin immunoprecipitation, we observed a decrease in the levels of Sp1, an increase in the levels of estrogen receptor, and no change in the levels of Sp3 in PS2 promoter in cells treated with 10 nM estradiol for 24 hours. This result suggests that Sp3 might have a structural function in the expression of the PS2 gene. Immunoprecipitation was carried out to identify proteins associated with Sp1 and Sp3, and Western blotting analysis shows that Sp1-HDAC1-ER and Sp3-HDAC1-ER complexes exit in T5 human breast cancer cells. In these human breast cancer T5 cells, both Sp1 and Sp3 are associated with DNA *in situ*, while the majority of Sp3 was tightly bound to the nuclear matrix, and a significant amount of Sp1 was not nuclear matrix protein. Since the differential distribution of Sp1 and Sp3 in breast cancer cells, we propose that Sp1 and Sp3 play a role in the PS2 gene transcription, however Sp3 may dominantly have a structural function in PS2 gene in human breast cancer cells. This result provides evidence that Sp1 family factors are not only involved in transcriptional regulation and also in the structural function.

Isolation of Proteins Cross-linked to DNA by Formaldehyde

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1. Introduction

Formaldehyde is a reversible cross-linker that will cross-link protein to DNA, RNA or protein (Orlando, 2000). Because of its high resolution (2 Å) cross-linking, formaldehyde is a useful agent to cross-link a DNA-binding protein of interest to DNA. For example, formaldehyde has been used to cross-link proteins to DNA in studies fine-mapping the distribution of particular DNA-binding proteins along specific DNA sequences (Dedon et al., 1991; Orlando, 2000).

When applied to a cell, formaldehyde will initially begin to cross-link protein to DNA. As the time of exposure to formaldehyde increases, proteins become cross-linked to one another. Soluble cellular components become more insoluble as they become cross-linked to one another and to the insoluble cellular material. Sonication is most commonly used to release cross-linked DNA -protein complexes from the insoluble nuclear material. Excessively cross-linking a cell with formaldehyde will cause nuclear DNA cross-linked to protein to become trapped within the insoluble nuclear material. Such an event will protect this cross-linked DNA from breakage by sonication. Moreover, the efficiency of formaldehyde DNA-protein cross-linking varies with cell type (Orlando et al., 1997). Therefore, two parameters must be considered when using formaldehyde as an agent for cross-linking DNA to a protein of interest: the release of DNA from the insoluble nuclear material after cross-linking, and the extent of sonication of cross-linked cells. The following chapter describes an approach for determining the optimal formaldehyde cross-linking conditions of a cell, and for isolating proteins cross-linked to DNA by formaldehyde.

2. Materials

All solutions are prepared from analytical grade reagents dissolved in double distilled water. Phenylmethylsulfonylflouride (PMSF) (1 mM) was added to all solutions immediately before use. All solutions were cooled on ice before use.

1. RSB Buffer: Add 10 ml of 1 M Tris-HCl, pH 7.5 (10 mM), and 2.5 ml of 4 M NaCl (10 mM) to approximately 800 ml of double-distilled water. Adjust the pH to 7.5 with NaOH then add 0.75 ml of 4 M $MgCl_2$ (3 mM). Re-adjust the pH if necessary, then make volume up to 1 L with double-distilled water.

2. Hepes Buffer: Add 2.38 g of Hepes (10 mM), and 2.5 ml of 4 M NaCl (10 mM) to 800 ml of double-distilled water. Adjust the pH to 7.5 with NaOH then add 0.75 ml of 4 M $MgCl_2$ (3 mM). Re-adjust the pH if necessary, then make volume up to 1 L with double-distilled water.

3. Lysis Buffer: Add 150 g urea (5 M), 95.5 g guanidine hydrochloride (2 M), and 58.5 g NaCl (2 M) to 16 ml of 1M KH_2PO_4 and 84 ml of 1M K_2HPO_4 (200 mM potassium phosphate buffer, pH 7.5). Stir while heating solution to approx. 50°C to speed up solubilization, make up to 500 ml with double distilled water, and filter solution with 1M Whatman filter.

4. Dounce Homogenizer (for 20 ml sample volume)

5. Hydroxyapatite Preparation: Weigh out 1 g of hydroxyapatite Bio-Gel® HTP Gel (Bio-Rad, CA) for every 4 mg of total cellular DNA in the cell lysate, as determined by A_{260} measurements. Place hydroxyapatite into a 30 ml polypropylene tube and pre-equilibrate the hydroxyapatite by suspending the resin in 6 volumes of lysis buffer. Gently invert the resin in the lysis buffer to mix, let the resin settle for approximately 20 min on ice, then decant the lysis buffer off the hydroxyapatite.

3. Methods

3.1 Formaldehyde Cross-linking of Immature Chicken Erythrocyte Nuclei

This procedure was performed on immature chicken erythrocytes isolated from Adult White Leghorn chickens treated with phenylhydrazine (Delcuve and Davie, 1989).

1. 2 ml of packed erythrocytes are resuspended in 15 ml of RSB buffer containing 0.25% NP-40.
2. The cells are homogenized 5 times in a dounce homogenizer and centrifuged at 1500 x g for 10 min at 4°C.
3. Steps 1 and 2 are performed two more times, leaving a pellet of nuclei.
4. The nuclei are resuspended in Hepes Buffer to an A_{260} of 20 U/ml.
5. Formaldehyde is then added to the suspension of nuclei to a final concentration of 1% (v/v).
6. The nuclei are mixed gently by inversion, and incubated for up to 15 min at room temperature.
7. After 0, 5, 10 and 15 min of formaldehyde cross-linking, 4 ml aliquots of nuclei are collected and made up to 125 mM glycine on ice to stop the cross-linking reaction.
8. The aliquots of nuclei are then centrifuged at 1500 x g for 10 min at 4°C.
9. The nuclear pellets are then washed in 10 ml of RSB buffer and centrifuged at 1500 x g for 10 min at 4°C.
10. The nuclear pellets are resuspended in 10 ml of ice-cold lysis buffer.

3.2 Sonication of Formaldehyde Cross-linked Cells

For each formaldehyde cross-linked sample from section 3.1:

1. The 4 ml of the lysed nuclei are transferred to a 50 ml Falcon tube and sonicated under 170 Watts for ten 30 second pulses with a Braun-sonic 1510 Sonicator. The sample is cooled on ice for 1 min waiting intervals between each pulse.

2. A 100 µl aliquot of nuclei is dialyzed against double distilled water (without PMSF) overnight at 4°C to remove excess urea and salt that may interfere with proteinase K digestion. The dialyzed sample is made to 0.5% SDS and 0.4 mg/ml proteinase K and incubated for 2 hours at 55°C to digest the protein.
3. The sample is then incubated at 65°C for 6 hours to reverse the formaldehyde cross-links between the DNA and protein.
4. The digested mixture is extracted 3 times with an equal volume of solution composed of phenol-chloroform-isoamyl alcohol in a 25:24:1 ratio, respectively.
5. To precipitate the DNA from the sample, 1/10th the sample volume of 3 M sodium acetate (pH 5.5) along with 3 volumes of absolute ethanol is added to the sample and the sample is incubated at -80°C for 20 min.
6. The sample is centrifuged at 12000 x g for 10 min at 4°C to pellet the DNA.
7. The DNA pellet is washed with 1 ml of ice-cold 70% ethanol, and centrifuged at 12000 x g for 10 min at 4°C.
8. The resulting DNA pellet is resuspended in 30 µl of double distilled water and 4 µl of this pellet is electrophoresed on a 0.8% agarose gel to identify high molecular weight DNA bands indicative of extensive cross-linking.

Section 3.3 Efficiency of Solubilization of Formaldehyde-Cross-linked Cells

For each formaldehyde cross-linked sample from section 3.1:

1. Transfer the 4 ml of cross-linked, lysed and sonicated nuclei to a 15 ml tube.
2. Determine the A_{260} of 10 µl of the total nuclear lysate.
3. Centrifuge the sample at 9000 x g for 10 min at 4°C.

4. The supernatant contains solubilized DNA and protein. Transfer the supernatant to a clean 15 ml tube.
5. Determine the A_{260} of 10 µl of the supernatant in 990 µl of lysis buffer.
6. Divide the A_{260} of the supernatant by the A_{260} of the total nuclear lysate and multiply this value by 100 to determine the percent of DNA released from the nuclei following formaldehyde cross-link and sonication.

Section 3.4 Isolation of Proteins Cross-linked to DNA by Formaldehyde

For each formaldehyde cross-linked sample from section 3.1:

1. Determine the approximate amount of DNA present in the 4 ml of lysed nuclei suspension from section 3.3.
2. Add the lysed nuclei suspension to pre-equilibrated hydroxyapatite (see notes) and mix by gentle inversion.
3. Incubate at 4°C for 1 hour on an orbitron.
4. Centrifuge the hydroxyapatite at 5000 x g for 5 min at 4°C.
5. Wash the hydroxyapatite with 10 ml of lysis buffer, mix by gentle inversion, and centrifuge at 5000 x g for 5 min at 4°C.
6. Repeat step 4 an additional two times.
7. Add 2 ml of lysis buffer to the washed hydroxyapatite and mix by gentle inversion.
8. Incubate this suspension at 68°C for 6 hours to reverse the formaldehyde cross-links between the DNA and protein.
9. Centrifuge the sample at 4°C or room temperature for 5 min at 5000 x g.
10. Place the supernatant containing protein that was cross-linked to DNA into pre-soaked dialysis

tubing.

11. Dialyze the sample overnight at 4°C against 2-3 L changes of double distilled water (include 0.5 mM PMSF in the first change).

12. Lyophilize the sample to a powder form.

13. Resuspend the powder in double distilled water and store at -20°C.

4. Notes

1. Sonication conditions will vary for different cell types.
2. Perform sonication on ice to avoid protein denaturation.
3. The extent of sonication depends on the length of the target DNA sequence. For example, if the association of a protein with a specific 1000 bp region needs to be determined, then the DNA should be sonicated to 500 bp to avoid the immunoprecipitation of DNA sequences surrounding the target region (see Kadosh and Struhl, 1998 for further explanation). However, if one is simply trying to determine if a protein of interest is associated with DNA, then the formaldehyde cross-linked cells need only be sonicated to an extent that allows the efficient release of nuclear DNA from the insoluble nuclear material (see note 5).
4. Formaldehyde reacts with amine groups of proteins. Thus, to ensure a high efficiency of cross-linking, it is important to resuspend the nuclei in a Hepes buffer before treatment with formaldehyde.
5. The duration of formaldehyde cross-linking will vary according to cell type, cell treatment and the degree to which the DNA associated with the nuclear material can be solubilized. For example, the sonication and subsequent centrifugation of a nuclear lysate may result in the solubilization of only 80% of total nuclear DNA. The acceptability of this percentage of DNA release from the nucleus depends on the location of the protein of interest. If the target protein is tightly associated with DNA that is associated with the nuclear matrix, the fraction of solubilized DNA-protein complexes may be somewhat depleted of the target protein even though as much as 80% of the nuclear DNA is released from the insoluble material.
6. Hydroxyapatite is a calcium phosphate resin that binds to the phosphate backbone of DNA.

7. A ratio of one gram of hydroxyapatite for every 4 mg of genomic DNA has been shown in our lab to bind all cellular DNA with 100% efficiency (data not shown).

8. The following equation can be used to determine the approximate amount of DNA within the cell lysate:

A_{260} x 50 µg/ml x 100 x (volume of cell lysate)/ 1000 µg per mg of DNA.

One A_{260} unit represents 50 µg of DNA per ml of cell lysate. To determine the µg of DNA in 1 ml of cell lysate, multiply the absorbance reading by 50 and then by the dilution factor (i.e. 100). Multiply the resulting concentration by the total volume of cell lysate to determine the total µg of DNA in the cell lysate. Divide the total µg by 1000 to convert this value into mg of DNA. The resulting amount of cellular DNA is only an approximation since some proteins within the cell lysate will have a peak absorption at 260 nm.

9. Gently inverting the hydroxyapatite resin when mixing avoids damaging the integrity of the resin.

10. The porosity of the dialysis tubing will depend on the size of the protein of interest.

11. Dialysis tubing should be soaked in distilled water for at least 30 min before use.

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Figure Legend

Figure 1. SDS-PAGE gel electrophoresis of total cellular proteins and formaldehyde DNA-cross-linked proteins. Formaldehyde cross-linked nuclei were separated into two fractions of equal volume. One fraction was cross-linked with formaldehyde for 0, 5, 10 and 15 min, and the DNA-cross-linked proteins were isolated by hydroxyapatite chromatography, dialyzed against double distilled water, lyophilized to a powder form and made up to a final volume of 200 µl with double-distilled water. The other fraction was lysed, dialysed, lyophilized to a powder form and made up to a final volume of 1 ml with double-distilled water. Equal volumes of total nuclear protein were loaded on to a 15% SDS-PAGE gel for each treatment. In addition, equal volumes of DNA-cross-linked proteins were loaded on to the same gel for each treatment. The gel was electrophoresed at 170 V for 70 min at room temperature, stained with coomassie blue overnight, and then destained. H3, H2B, H2A and H4 represent histones H3, H2B, H2A and H4, respectively.

Isolation of Proteins Cross-linked to DNA by Cisplatin

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1. Introduction

One way of identifying and further characterizing transcription factors is to study their association with DNA *in situ*. Many studies have performed this task using agents such as formaldehyde that cross-link proteins to DNA. However, the treatment of cells with agents such as formaldehyde results in the cross-linking of protein to DNA, and protein to protein. Thus, proteins cross-linked to DNA-binding proteins may be misinterpreted as DNA-binding proteins. To overcome this obstacle, researchers have focussed their attention on cisplatin (*cis*-DDP; *cis*-platinum (II)diamminedichloride), a cross-linking agent shown to cross-link protein to DNA and not to protein (Foka and Paoletti, 1986). Recent studies have shown that the majority of proteins cross-linked to DNA by cisplatin *in situ* are nuclear matrix proteins (Davie et al., 1998, Ferraro et al., 1995 and 1996). We have also shown that cisplatin cross-links nuclear matrix-associated transcription factors and cofactors to DNA in the MCF-7 human breast cancer cell line (Samuel et al., 1998). Thus, cisplatin appears to be an effective cross-linking agent for studying the role of transcription factors and nuclear matrix proteins in transcription. In support of this, we have discovered cisplatin DNA-cross-linked nuclear matrix (NM) proteins whose levels vary between well and poorly differentiated human breast cancer cell lines (Spencer et al., 2000). Such changes in protein levels indicate that breast cancer development most likely involves changes in DNA organization, and, likely, changes in transcriptional events. Currently used nuclear matrix protein extraction protocols have been effective in identifying diagnostic NM protein markers for bladder cancer detection (Konety et al., 2000). Thus, the isolation of cisplatin DNA-cross-linked proteins is a complementary approach to these nuclear matrix extraction protocols that may also be useful in the detection of additional nuclear matrix proteins for cancer diagnosis.

The following chapter provides a detailed description of the isolation of proteins cross-linked to DNA by cisplatin. This method is similar to that previously published by Ferraro and colleagues (1991). In addition, this method has been successfully performed on human breast cancer cells and avian erythrocytes.

2. Materials

All solutions are prepared fresh from analytical grade reagents dissolved in double distilled water. Phenylmethylsulfonylflouride (PMSF) (1 mM) was added to all solutions immediately before use. With the exception of the cisplatin solution, all solutions are cooled on ice before use.

1. **Hanks buffer:** 0.2 g KCl (5.4 mM), 0.025 g Na_2HPO_4 (0.3 mM), 0.03 g KH_2PO_4 (0.4 mM), 0.175 g $NaHCO_3$ (4.2 mM), 0.07 g CaCl (Ann) (1.3 mM), 62.5 µl 4 M MgCl (0.5 mM),0.075 g $MgSO_4$ (0.6 mM), 4 g NaCl (137 mM), and 0.5 g D-glucose (5.6 mM) in 500 ml of double distilled water.

2. **Hanks buffer with Na Acetate instead of NaCl:** Refer to recipe for Hanks buffer except replace the NaCl with 9.3 g sodium acetate (137 mM).

3. **1 mM Cisplatin Cross-linking Solution:** Add 0.003 g of cisplatin (*cis*-platinum (II)-diammine dichloride; Sigma) to 10 ml of Hanks buffer containing sodium acetate instead of NaCl. Cover the solution with foil to protect the cisplatin from the light and stir over gentle heat (approx. 40°C) to dissolve the cisplatin. Once the cisplatin is dissolved, keep the solution at room temperature in a foil-covered or amber bottle.

4. **Lysis Buffer:** Add 150 g urea (5 M), 95.5 g guanidine hydrochloride (2 M), and 58.5 g NaCl (2 M) to 16 ml of 1M KH_2PO_4 and 84 ml of 1M K_2HPO_4 (200 mM potassium phosphate buffer, pH 7.5). Stir while heating solution to approx. 50°C to speed up solubilization, make up to 500 ml with double distilled water, and filter solution with 1M Whatman filter.

5. **Hydroxyapatite Preparation:** Weigh out 1 g of hydroxyapatite Bio-Gel® HTP Gel (Bio-Rad, CA) for every 4 mg of total cellular DNA in the cell lysate, as determined by A_{260} measurements. Place hydroxyapatite into a 30 ml polypropylene tube and pre-equilibrate the

hydroxyapatite by suspending the resin in 6 volumes of lysis buffer. Gently invert the resin in the lysis buffer to mix, let the resin settle for approximately 20 min on ice, then decant the lysis buffer off the hydroxyapatite.

6. **Reverse Lysis Buffer**: Add 3.8 g Thiourea (1 M), 9.55 g Guanidine Hydrochloride (2 M), and 5.85 g NaCl (2 M), to 1.6 ml 1 M KH_2PO_4 and 8.4 ml 1 M K_2HPO_4 (200 mM potassium phosphate buffer, pH 7.5). Stir while heating solution to approx. 50°C, make up to 50 ml with double distilled water.

3. Methods

Isolation of Cisplatin DNA-Cross-linked Proteins

1. Rinse 1 x 10^7 cells in 30 ml of cold Hanks buffer.
2. Centrifuge at 50 x g for 5 min at room temperature.
3. Repeat the rinse 2 more times.
4. Decant the Hanks buffer from the cell pellet and add 10 ml of 1 mM Cisplatin solution to the pellet.
5. Incubate at 37°C for 2 hour with shaking.
6. Centrifuge at 50 x g for 5 min at room temperature.
7. Resuspend the cell pellet in 10 ml of cold lysis buffer, and store on ice.
8. Measure the A_{260} of 10 µl. of the cell lysate and use this value to determine the grams of hydroxyapatite required for DNA isolation.
9. Pre-equilibrate the hydroxyapatite in a 30 ml tube.
10. Transfer the cell lysate into the tube containing the pre-equilibrated hydroxyapatite, and mix by gentle inversion until all the hydroxyapatite is resuspended.

11. Incubate 1 hr at 4°C on an orbitron.

12. Centrifuge at 5000 x g for 5 min at 4°C.

13. Remove the supernatant which contains proteins not cross-linked to DNA.

14. Wash the hydroxyapatite resin with 20 ml of ice-cold lysis buffer by gentle inversion until the resin is completely resuspended.

15. Centrifuge the washed resin at 5000 x g for 5 min at 4°C.

16. Repeat this wash 2 more times.

17. Add 10 ml of cold reverse lysis buffer to the hydroxyapatite resin.

18. Incubate at 4°C for 2 hours on the orbitron to reverse the cross-link between the protein and resin-bound DNA.

19. Centrifuge the hydroxyapatite resin at 5000 x g for 5 min at 4°C.

20. Carefully remove the supernatant and place it in dialysis tubing that has been soaked in distilled water for at least 30 min.

21. Dialyze the protein sample at 4°C against four 2 L changes of double distilled water over a 24 hour period. Include 0.5 mM PMSF in the first change of double distilled water.

22. Transfer the dialyzed solution from the dialysis tubing to a 13 ml centrifuge tube, and freeze at -80°C until solution is completely frozen.

23. Lyophilize the solution to a dry powder.

24. Resuspend the dry powder in 100 µl of 8 M urea and store at -20°C.

4. Notes

1. The conditions for cisplatin cross-linking and protein isolation may vary for other cell types.
2. NaCl is excluded from the cross-linking solution since chloride ions impair the efficiency of the cross-linking reaction by competing with cisplatin for cellular proteins (Lippard, 1982).
3. Human breast cancer cells treated with 1 μM cisplatin display a drastic decrease in cell number after two hours. Thus, a prolonged incubation time (i.e. greater than 2 hours) in the presence of cisplatin may result in the activation of pro-apoptotic proteins involved in protein degradation (Samuel et al., 1998).
4. Filtering the lysis solution with 1 M Whatman filter paper will remove particulates that may interfere with A_{260} measurements.
5. Hydroxyapatite is a calcium phosphate resin that binds to the phosphate backbone of DNA.
6. One gram of hydroxyapatite is used for every 4 mg of genomic DNA in the cell lysate, as determined by A_{260} measurements, since this ratio has been shown previously in our lab to bind all cellular DNA with approximately 100% efficiency (data not shown).
7. Gentle inversion of the hydroxyapatite resin is important to avoid damaging the integrity of the resin.
8. For measuring the A_{260} of the cell lysate, transfer 10 μl of cell lysate into a tube containing 990 μl of lysis buffer.
9. The porosity of the dialysis tubing will depend on the size of the protein of interest.
10. Dialysis tubing should be soaked in distilled water for at least 30 min before use.
11. For determining an approximate amount of total cellular DNA within the lysate, the following equation is used:

A_{260} x 50 µg/ml x 100 x 10 ml/ 1000 µg per mg of DNA.

One A_{260} unit represents 50 µg of DNA per ml of cell lysate, thus the absorbance reading is first multiplied by 50 and then by the dilution factor (i.e., 100) to determine the µg of DNA in 1 ml cell lysate. The resulting value is multiplied by the total volume of cell lysate (i.e., 10 ml) to determine the total µg of DNA in the cell lysate and then divided by 1000 to convert this value into mg of DNA. The determined amount of cellular DNA is only considered an approximation since the cell lysate contains some proteins with a peak absorption at 260 nm.

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Figure Legend

Figure 1. Two-dimension gel electrophoresis profile of cisplatin DNA-cross-linked proteins. Proteins (30 μg) cross-linked to DNA *in situ* by cisplatin in MCF-7 human breast cancer cells were electrophoretically resolved on a two dimension gel, and the gel was stained with silver. K8, K18 and K19 represent cytokeratins 8, 18 and 19, respectively. The transcription factor hnRNPK is designated as hK. The position of the carbamylated forms of carbonic anhydrase is indicated by ca. The position of the molecular weight standards (in thousands) is shown to the left of the gel pattern.